

RESEARCH ARTICLE

Antagonistic effect of volatile and non-volatile compounds from *Streptomyces* strains on cultures of several phytopathogenic fungi

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ABSTRACT

Fungi and oomycetes are important plant pathogens that constantly attacked plants, thus compromising the production of foods worldwide. *Streptomyces* strains might be useful to control fungal pathogens by different mechanism. The *in vitro* antagonistic activity of non-volatile and volatile metabolites from four *Streptomyces* strains was evaluated over cultures of phytopathogenic fungi and oomycetes. The non-volatile compounds from *Streptomyces* strains significantly reduced (44.2 to 92.1%) the growth of aerial mycelium of pathogens. The volatile compounds (VOCs) from *Streptomyces* strains reduced both aerial mycelium (22.5 to 96.7%) and mycelium growing inside of culture medium (0.0 - 9.4%). The pathogens maintained their capacity to grow normally in fresh culture medium without antagonists after confrontations with antagonist VOCs. The analysis of VOCs by gas chromatography coupled to mass spectrometry revealed different kinds of VOCs included alcohols, aldehydes, ketones, esters, terpenes, terpenoids, thioethers, among others. The most abundant VOCs were *trans*-1,10-dimethyl-*trans*-9-decalol (geosmin), 2-methylisoborneol, 2-methyl-2-bornene, 1,4-dimethyladamantane, and 4-penten-1-ol, trifluoroacetate. The antipathogenic activity of nine pure VOCs that had been identified in cultures of the *Streptomyces* strains alone was evaluated *in vitro* against phytopathogenic fungi and oomycetes. *Trans*-2-hexenal was the most effective of these VOCs, inhibiting completely the growth of tested phytopathogens. The volatile and non-volatile compounds from *Streptomyces* strains effectively reduced the *in vitro* growth of phytopathogens and they might be used as biological control. Further studies are required to demonstrate this activity on open field conditions.

Keywords: Antibiosis; Beneficial microorganisms; Biological control; Fungal phytopathogens; Oomycetes

INTRODUCTION

Plants are constantly attacked by a wide diversity of phytopathogenic microorganisms, causing important crop losses in preharvest ($\geq 20\%$) and postharvest ($\geq 10\%$) (Bebber and Gurr, 2015). The annual cost of these losses worldwide has been estimated to be of billions of dollars (Sikes et al., 2018). Fungi and oomycetes are important plant pathogens, compromising significantly the production of foods worldwide (Möller and Stukenbrock, 2017). These complications are not new, and phytopathogens have been a widespread problem for a long time, causing negative impacts on availability of plant foods (Fisher

et al., 2012). The control of these pathogens is a priority in agriculture and has mainly been based on chemical fungicides, however, the negative impacts of these compounds on the environment, humans, agriculture, and ecology have forced to search for biological control agents, which do not exert the negative effects regarded to chemicals (Marian and Shimizu, 2019; Pliego et al., 2011). Actinomycetales is family of filamentous bacteria (Gram-positive) widely known for the its antimicrobial activity (Sharma et al., 2014). The *Streptomyces* genus belongs to this family, and is widely distributed in nature, especially in soil, and produces a large number of secondary metabolites with antimicrobial activity widely used in agriculture and

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industry (Dhanasekaran et al., 2009; Sidda and Corre, 2012; Todosiichuk et al., 2015). This antibiosis have been attributed to non-volatile and volatile compounds (VOCs), although the volatile antibiotics have received little attention (Scholler et al., 2002). Several non-volatile antimicrobial compounds from *Streptomyces* against phytopathogenic fungi have been identified, including: valinomycin (Park et al., 2008), reveromycins A and B (Lyu et al., 2017), among others. *Streptomyces* strains also produce many types of VOCs, including alkanes, alkenes, aromatic hydrocarbons, alcohols, sulfides, ketones, esters, terpenes, among others (Boukaew et al., 2013; Wan et al., 2008). These VOCs are important for the interaction between microorganisms and suppression of pathogens (Guevara-Avendaño et al., 2019). Several phytopathogens such as *Penicillium italicum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium moniliforme*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* have successfully been suppressed by VOCs from *Streptomyces* strains (Boukaew et al., 2013; Li et al., 2010, 2012; Wang et al., 2013; Wu et al., 2015). Recently, Yang et al. (2019) demonstrated that the VOCs from *S. albobiflavus* TD-1 reduced the growth and production of aflatoxins of *Aspergillus flavus*. Additionally, the *Streptomyces* genus produces plant-growth promoting compounds, including indole-3-acetic acid (auxin), griseobactin (siderophore), and gibberellic acid (de Jesus Sousa and Olivares, 2016; Dias et al., 2017). *Streptomyces* strains might be useful for controlling pathogens of crops and increase the productivity of plants (Boukaew et al., 2013). Thus, the aims of the study were as follows: (i) to evaluate the *in vitro* antagonistic activity of four *Streptomyces* strains against selected phytopathogenic fungi and oomycetes by non-volatile and volatile metabolites, (ii) to identify the volatile compounds emitted by *Streptomyces* strains which may be involved in mycelial growth suppression and (iii) to evaluate the efficacy of individual volatile compounds on the suppression of phytopathogenic fungi.

MATERIALS AND METHODS

Reagents and microorganisms

All reagents (analytical grade) and standards of VOCs were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Four *Streptomyces* strains (*S. cangkringensis* CIAD-CA07, *S. misionensis* CIAD-CA27, *S. kanamyceticus* CIAD-CA45, and *S. kanamyceticus* CIAD-CA48) used in the study were isolated from maize (corn, *Zea mays* L.), bean (*Phaseolus vulgaris* L.), and apple (*Malus domestica* Borkh.) crops, respectively, in Chihuahua, Mexico and were preserved on International Streptomyces Project Medium Number 4 agar (ISP4) at 4 °C. The fungal pathogens *Fusarium solani* (FSO) and *Alternaria alternata* (AAL) obtained from the ceparium of the Centro de Investigación en Alimentación

y Desarrollo A.C. (Campus Cuauhtémoc, Chihuahua) were isolated from tomato (*Solanum lycopersicon* L.) and apple (*Malus domestica* Borkh.) crops in Chihuahua, Mexico. These fungal isolates were morphologically identified based on cultural and microscopic characteristics as described by Watanabe (2010), and then, corroborated by Dra. Sylvia Patricia Fernández-Pavía (Universidad Michoacana de San Nicolás de Hidalgo, México-UMSNH). Oomycetes *Phytophthora capsici* (PHC) strain CPV-283 (AR26) isolated from pepper (*Capsicum annum* L.) in Yurécuaro, Michoacán, Mexico; *Pythium aphanidermatum* (PYA) isolated from papaya (*Carioca papaya* L.) from Pescadero, Baja California Sur, Mexico; and *Phytophthora vexans* (PPV) from avocado (*Persea americana* Mill.) in Xalisco, Nayarit, México were provided by Dra. Fernández-Pavía obtained from a collection belonging to the Instituto de Investigaciones Agropecuarias y Forestales of the UMSNH. These isolates were selected based on their pathogenic capacity documented in previous studies (Castro-Rocha et al., 2016; Rodríguez-Alvarado et al., 2001). The fungal isolates *F. solani* and *A. alternata* were maintained on Potato Dextrose Agar (PDA) at 4 °C. The oomycetes *P. capsici*, *P. aphanidermatum*, and *P. vexans* were maintained on Corn Meal Agar (CM). The microorganisms were sub-cultured on fresh media at 28 ± 1 °C for 5-7 d previous to be used.

Molecular identification of *Streptomyces* isolates

The identification of *Streptomyces* strains was based on the analysis of the partial gene sequence encoding 16S rRNA from genomic DNA extracted according to Ruiz-Cisneros et al. (2017). To amplify the region of 16S rRNA gene, fragment was prepared by PCR amplification using the high guanine-cytosine bacteria oligonucleotides F243 (5'-GGATGAGCCCGCGGCCTA-3') and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (Duthoit et al., 2003). These PCR products were examined by electrophoresis on a 1% agarose gel and then, were sequenced at Macrogen Company (Rockville, MD, USA). The obtained sequences were deposited to NCBI database (GenBank). Further phylogenetic analysis by maximum likelihood method was performed using the MEGA X software (Kumar et al., 2018), and Tamura 3-parameter model with values based on 1,000 bootstrapping replicates.

Antagonistic activity of non-volatile compounds from *Streptomyces*

The antifungal activity of *Streptomyces* strains was assessed against five pathogens by dual culture method in Petri dishes of 90 mm of diameter containing Czapek Dox Agar (CDA) (Fig. 1). Circle explants (7 mm of diameter) of each isolate were placed on four equidistant points of a Petri dish and incubated at 28 ± 1 °C for 10 d, according to Dávila-Medina et al. (2013). The radial growth of the pathogens was measured every 24 h until fungi and oomycetes filled

the control Petri dishes. The inhibition of the radial growth (IRG) was determined using the formula reported by Evangelista-Martínez et al. (2020). The bioassays included nine replicates for each confrontation.

Antifungal activity of volatiles from *Streptomyces*

The *Streptomyces* strains were grown on cooked-sterile wheat grains incubated for 10 d described by Wan et al. (2008) and Boukaew et al. (2013) with modifications. Then, 15 g of inoculated wheat were placed on Petri dishes (90 mm diameter). On the other hand, a circle explant (7 mm of diameter) containing mycelium and spores of the pathogen was placed at the center of another Petri dish containing CDA medium. Both Petri dishes were opened and placed face to face according to Yang et al. (2019). Control Petri dishes only contained sterile wheat grains (Fig. 1). The dishes were incubated at 28 ± 1 °C and the colony diameter of each dish was measured systematically every 24 h. The IRG was calculated as indicated above. A poor aerial mycelium growth was observed, compared to mycelium inside of the medium in this assay. In both, the mycelial growth was measured using a digital caliper (KNOVA, Mexico) under a stereomicroscope (Leica G26). Thus, explants of these mycelia were placed in Petri dishes with PDA in absence of *Streptomyces* and incubated at the same conditions indicated above to determine if they were able to recover their normal growth rate.

Analysis of volatile compounds by GC-MS

The analysis of VOCs from *Streptomyces* was carried out according to Wan et al. (2008), with modifications. After 10 d of incubation, 100 mL of saturated sodium chloride sterile solution were added to the 250 mL bottles containing wheat grains (30 g) inoculated with *Streptomyces*.

Control treatments contained cooked-sterile wheat without *Streptomyces* strains. The mixtures were stirred at 400 rpm at 60 °C for 1 h. The VOCs were collected during 40 min by a DVB/CAR/PDMS fiber (50/30 µm film thickness) and analyzed by gas chromatography coupled to mass spectrometry (VARIAN SATURN 2100D, CA, USA). The samples were injected in the splitless mode. The VOCs were continuously desorbed at 240 °C and separated in a Hewlett Packard HP-5 column (30 m × 0.25 mm, 0.25 µm). The initial temperature of the oven was 40 °C, which was kept constant for 2 min. Then, the oven temperature increased to 150 °C at a rate of 2 °C/min, then at 280 °C at a rate of 100 °C/min remaining at such temperature for 2 min. Helium gas was used as carrier at a flow rate of 1 mL/min. The mass spectra were in the range of m/z of 35 to 500. The identification was performed by comparing the MS spectra with those of the National Institute of Standards and Technology (NIST) database. These experiments were performed in triplicate.

Antagonistic activity of pure VOCs against phytopathogens

The antifungal activity of pure VOCs: 2-pentanone, 2-(methylthio) ethanol, *trans*-2-hexenal, 2,5- dimethylfuran, α -pinene, 2-methyl-3-pentanone, 3-methyl-2-butanol, geosmin + 2-methylisoborneol, valencene and dimethyl disulfide (positive control) were individually assessed *in vitro* against pathogens according to the method of Yang et al. (2019). The concentration of the volatiles was adjusted to 100 µL·L⁻¹. The IRG was calculated as indicated above. Filter papers without VOCs were used as control treatments.

Data analysis

The experiment was performed under a completely randomized design with three replicates. The data were analyzed by an analysis of variance (ANOVA) and an analysis of means by the Tukey test ($p=0.05$). The IRG values were studied by principal component analysis.

RESULTS

Molecular identification of *Streptomyces* isolates

The analysis of the partial 16S rRNA gene sequence revealed high similarities of *Streptomyces* isolates with identities > 99 % compared to those deposited in the NCBI/BLAST database. Based on molecular characters and phylogenetic analysis of 16S rRNA gene *Streptomyces* strains were identified as *S. cangkringensis* CIAD-CA07, *S. misionensis* CIAD-CA27, *S. kanamyceticus* CIAD-CA45, and *S. kanamyceticus* CIAD-CA48) (Fig. 2) and DNA sequences were deposited in GenBank under accession numbers MK968576.1, MK968589.1, MK968601.1, and MK968603.1, respectively.

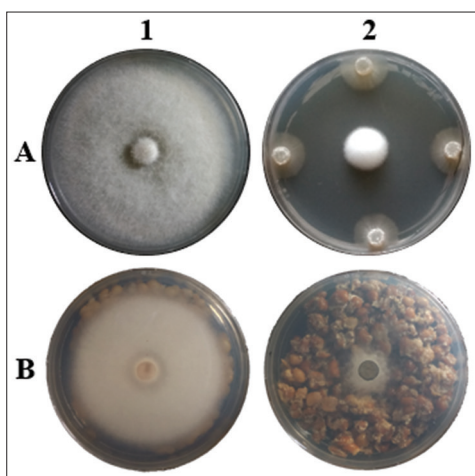


Fig 1. In vitro inhibition of mycelial growth of plant pathogenic fungi by *Streptomyces* strains. (A1 and B1) Mycelial growth of fungal control on CDA medium and wheat grains, respectively. (A2 and B2) Antifungal activity of non-volatile compounds by dual culture method and volatile compounds using two inverse face-to-face Petri Dishes.

Antagonistic activity of non-volatile compounds from *Streptomyces*

The *Streptomyces* strains significantly reduced the IRG values of tested pathogens, according to the outcome of dual confrontation assays (Fig. 3). This reduction ranged from 44.69 to 78.38% for *A. alternata*, from 44.24 to 86.50% for *F. solani*, from 70.76 to 92.10% for *P. capsici*, from 52.25 to 79.95% for *P. vexans* and from 60.67 to 88.51% for *P. aphanidermatum*. The highest IRG was caused by *S. kanamyceticus* CIAD-CA45 against *P. capsici* (92.10%), while the lowest values (44.69 and 44.24%) were caused by *S. kanamyceticus* CIAD-CA48 against *A. alternata* and *F. solani*, respectively. The principal

component analysis (Fig. 4) showed that the strains *S. cangkringensis* CIAD-CA07 and *S. misionensis* CIAD-CA27 significantly decreased the growth of phytopathogenic fungi (*F. solani* and *A. alternata*), while the strains *S. kanamyceticus* CIAD-CA45 and *S. kanamyceticus* CIAD-CA48 mostly reduced the growth of phytopathogenic Oomycetes (*P. capsici*, *P. aphanidermatum* and *P. vexans*).

Antifungal activity of volatiles from *Streptomyces*

The pathogens exposed to VOCs emitted by *Streptomyces* strains apparently did not develop aerial mycelium on the culture medium, however, when observing under

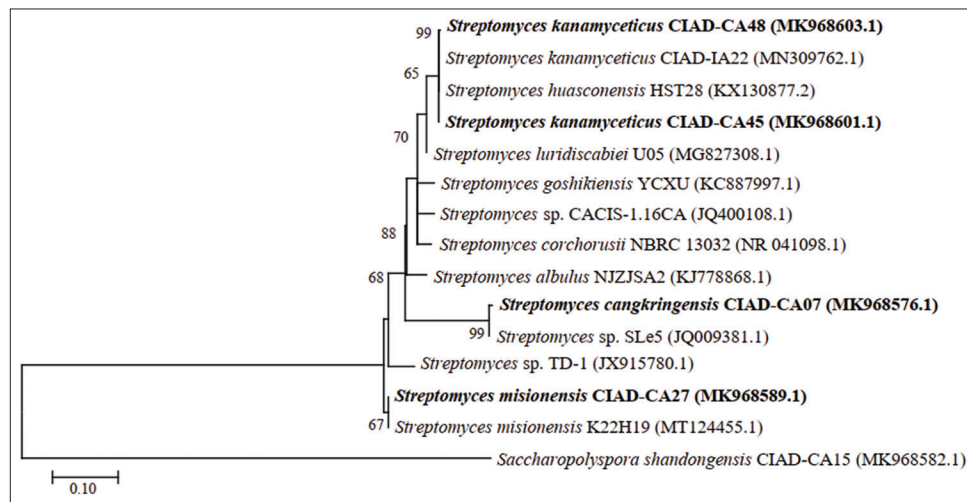


Fig 2. Phylogenetic tree of maximum likelihood and Tamura 3-parameter model based on 16S rRNA gene region sequences showing phylogenetic affinities of *Streptomyces* isolates belonging to *S. kanamyceticus*, *S. cangkringensis* and *S. misionensis*. Strains in this study are highlighted in bold. Support values exceeding 50%, are indicated at the nodes as percentage values based on 1,000 bootstrap replicates. Scale bar corresponds to 0.10 nucleotide substitutions per site. The tree is rooted with *Saccharopolyspora shandongensis* CIAD-CA15.

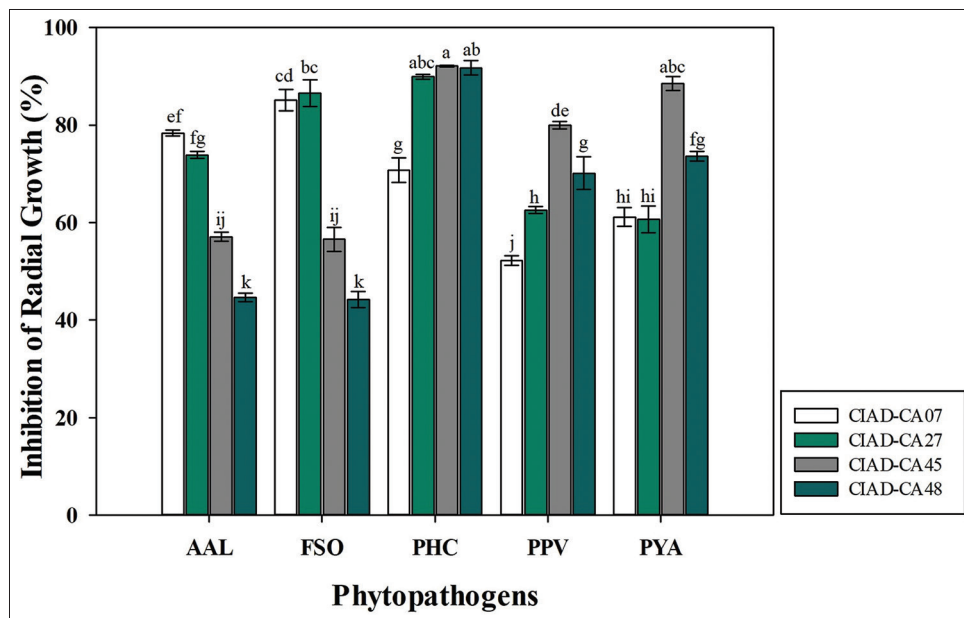


Fig 3. Inhibition of radial growth (IRG) of common phytopathogens by *Streptomyces* strains by dual confrontation. Phytopathogens: *Alternaria alternata* (AAL), *Fusarium solani* (FSO), *Phytophthora capsici* (PHC), *Phytophthora vexans* (PPV), and *Pythium aphanidermatum* (PYA). Mean values connected by same letters are statistically similar. Slim upper bars indicate the standard deviation of means.

the stereomicroscope a sparse mycelium inside of the culture medium was observed (Fig. 5), therefore, both aerial mycelium and mycelium inside of the medium were measured. The IRG values for aerial mycelium ranged from 81.29 to 96.49% for *A. alternata*, from 64.74 to 96.74% for *F. solani*, from 48.11 to 80.35% for *P. capsici*, from 22.58

to 59.84% for *P. vexans*, and from 52.87 to 96.26% for *P. aphanidermatum* (Fig. 6). The VOCs from *Streptomyces* strains were less effective to reduce the growth of mycelium inside of the medium, with IRG ranging from 0.00 to 7.51% for *A. alternata*, from 1.24 to 9.39% for *F. solani*, from 0.00 to 4.08% for *P. capsici*, from 0.00 to 7.38% for *P. vexans*, and from 0.00 to 0.93% for *P. aphanidermatum* (Fig. 6). All samples of aerial mycelium and mycelium growing inside of the medium recovered their normal growth rate after being placed in fresh PDA without *Streptomyces* strains.

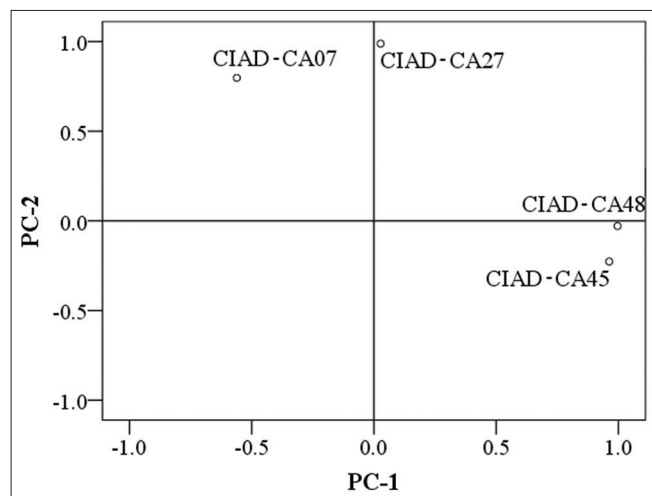


Fig 4. Principal component analysis for inhibition data of *Streptomyces* strains against common phytopathogens by dual confrontation. Scores (PC-1 and PC-2) for the different values of IRG are showed with the loadings.

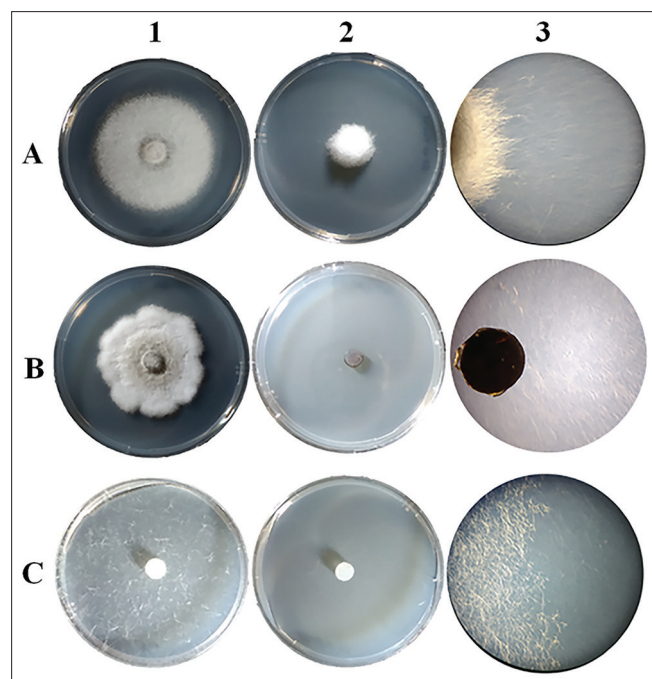


Fig 5. Types of mycelial fungal growth induced by volatile compounds emitted by *Streptomyces* strains. 1A-1C) Fungal controls (1A: *Fusarium solani*, 1B: *Alternaria alternata*, 1C: *Pythium aphanidermatum*). In vitro confrontations: 2A-2C) Showed aerial growth and 3A-3C) growth beneath the culture medium on *F. solani*, *A. alternata* and *P. aphanidermatum* in presence of *Streptomyces* *cangkringensis* CIAD-CA07, *S. misionensis* CIAD-CA27 and *S. kanamyceticus* CIAD-CA45 respectively.

Analysis of volatile compounds by GC-MS

The number of VOCs identified in the headspace of bottles containing wheat inoculated with *S. cangkringensis* CIAD-CA07, *S. misionensis* CIAD-CA27, *S. kanamyceticus* CIAD-CA45 and *S. kanamyceticus* CIAD-CA48 was 22, 26, 15, and 10, respectively (Table 1). The volatile profile depended of the strain. These VOCs were alcohols, aldehydes, ketones, esters, terpenes, terpenoids, thioethers, among others. *S. cangkringensis* CIAD-CA07 mainly produced 2-methylisoborneol and 2-methyl-2-bornene. The most abundant volatile observed in cultures of *S. misionensis* CIAD-CA27 were *trans*-1,10-dimethyl-*trans*-9-decalol (geosmin) and 1,4-dimethyladamantane, while *S. kanamyceticus* CIAD-CA48 mainly produced 4-penten-1-ol, trifluoroacetate. The *S. kanamyceticus* strain CIAD-CA45 showed a low production of VOCs (Table 1).

Antagonistic activity of pure VOCs against phytopathogens

Pure VOCs caused different IRGs (Table 2) and only *trans*-2-hexenal completely inhibited the mycelial growth of all pathogens at a concentration of 100 μL^{-1} . Dimethyl disulfide (positive control) caused IRGs ranged from 26.29 to 99.93%. The IRGs for the other pure VOCs ranged from 0.9 to 63.4%. *Phytophthora capsici* was the most susceptible pathogen to tested VOCs.

DISCUSSION

Phylogenetic analysis of the *Streptomyces* isolates showed separations within species related and some others selected for their ability to produce antimicrobial metabolites (Fig. 2). These variations might be related to the genetic variability, which cause differences in the morphology, metabolism and antifungal activity (Quecine et al., 2008). In this regard, Al_husnan and Alkahtani (2016) mentioned that although several *Streptomyces* species are closely related, there are strains among the same species capable of producing different antifungal secondary metabolites.

Tested *Streptomyces* strains reduced the growth of pathogens when both microorganism types shared the same culture

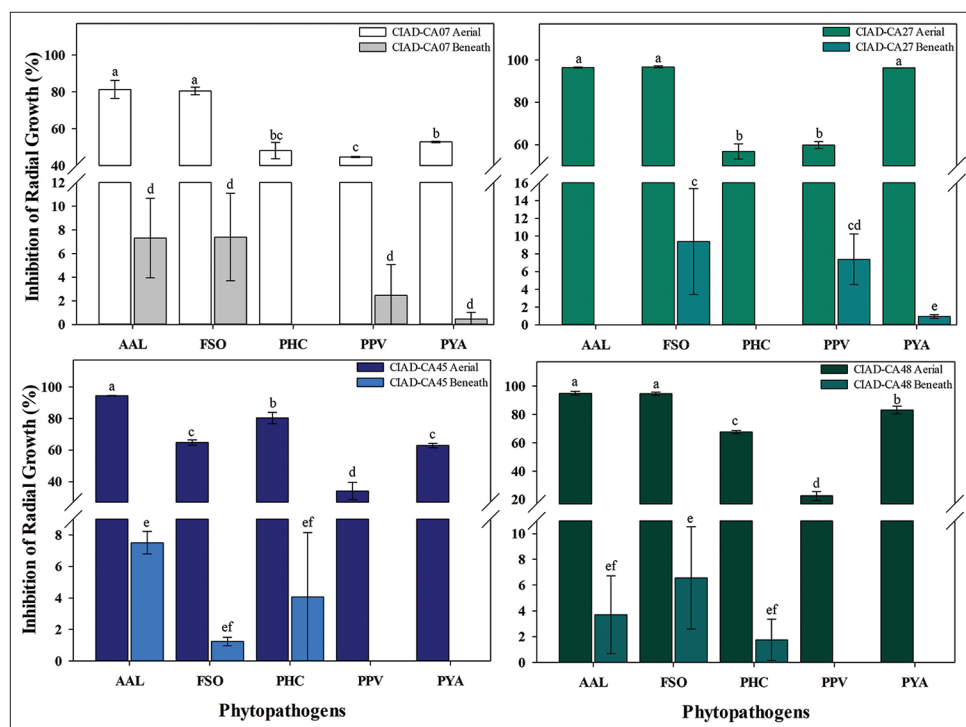


Fig 6. Inhibition of radial growth (IRG) of common phytopathogens by volatile from *Streptomyces* strains. Aerial mycelium and mycelium inside of the medium. Pathogens: *Alternaria alternata* (AAL), *Fusarium solani* (FSO), *Phytophthora capsici* (PHC), *Phytophthora vexans* (PPV), and *Pythium aphanidermatum* (PYA). Mean values connected by same letters are statistically similar. Slim upper bars indicate the standard deviation of means.

Table 1. Volatile compounds produced by *Streptomyces* strains (*S. cangkringensis* CIAD-CA07, *S. misionensis* CIAD-CA27, *S. kanamyceticus* CIAD-CA45 and *S. kanamyceticus* CIAD-CA48).

Possible volatile compound	Retention time (min)	Relative peak area (%)			
		CA07	CA27	CA45	CA48
2-Butanone, 3-methyl-	2.295	--	--	0.283	--
2-Butanol, 3-methyl-	2.515	--	--	0.425	--
Hexanoic acid, 2-ethyl-3-hydroxy-, methyl ester	2.568	--	0.099	--	--
2-Pentanone	2.591	0.086	--	0.316	--
Furan, 2,5-dimethyl-	2.826	--	0.143	--	--
4-Penten-1-ol, trifluoroacetate	3.215	--	0.35	--	7.816
Oxirane, (2-methylbutyl)-	3.36	--	--	1.561	--
Heptane	3.569	--	--	3.217	--
2-Pentanone, 3-methyl-	3.595	--	--	--	0.791
3-Pentanol, 2-methyl-	4.008	--	0.262	0.278	--
Butanoic acid, 2-methyl-, methyl ester	4.166	--	1.485	--	--
3-Pentanone, 2-methyl-	4.392	--	--	0.395	--
2-Propenoic acid, 4-methylpentyl ester	4.46	0.092	--	--	--
2-Oxepanone, 7-methyl	4.47	--	--	0.794	0.554
Propanoic acid, 2-methoxy-, methyl ester	4.556	--	0.14	--	--
2-(methylthio) ethanol	5.971	--	--	--	0.623
<i>trans</i> -2-Hexenal	5.981	--	0.262	--	--
3-Pentanone, 2,2-dimethyl	5.983	--	--	0.644	--
Cyclopentanol, 2-methyl-	6.084	--	0.435	--	--
(S)-3,4-Dimethylpentanol	7.195	--	0.409	--	--
2,5-Dimethylhex-5-en-3-yn-2-ol	7.879	--	0.321	--	--
Pentanoic acid, 2,4-dimethyl-, methyl ester	9.492	--	0.719	--	--
(1R)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene	9.778	0.097	--	--	--
1,3-Benzenediol, 4-ethyl-	10.229	--	0.183	--	--
3-Hexen-2-one, 3,4-dimethyl-	11.43	--	--	2.535	--

(Contd...)

Table 1. (Continued)

Possible volatile compound	Retention time (min)	Relative peak area (%)			
		CA07	CA27	CA45	CA48
Bicyclo[3.1.0]Hexane, 4-methylene-1-(1-methylethyl)-	12.01	0.209	--	--	--
Benzene, 1-ethoxy-4-ethyl-	12.182	1.234	--	--	--
2-Methylenebornane	12.474	1.326	--	--	--
3-Octene-2,6-dione, 5,5,7-trimethyl-, (E)-	13.422	--	0.253	--	--
2-Methyl-2-bornene	14.323	5.418	--	--	--
Pentanoic acid, 3-hydroxy-2-methyl-, methyl ester	14.48	--	0.475	--	--
3-Penten-2-one	14.608	--	0.421	--	--
Hexanoic acid, 2 ethyl-, methyl ester	16.553	--	--	0.435	--
2-Cyclopenten-1-one, 2-(2-butenyl)-4-hydroxy-3-methyl-, (Z)-	22.754	--	--	1.006	--
Benzenamine, 2,3,4,5,6-pentamethyl-	24.191	--	--	0.549	--
2-Methylisoborneol	25.185	40.465	--	--	--
1,4-Dimethyladamantane	27.687	1.556	12.927	--	--
1H-Idene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)-	29.166	--	1.168	--	--
3,4-Dimethoxytoluene	29.38	--	--	--	0.292
1,3a-Ethano-3aH-indene, 1,2,3,6,7,7a-hexahydro-2,2,4,7a-tetramethyl-, [1R-(1.alpha.,3a.alpha.,7a.alpha.)]-	33.532	0.295	--	--	--
1-Ethyl-3-(propen-1-yl) adamantane	34.221	0.255	--	--	--
.beta.-Neclovene	35.598	2.793	--	--	--
.beta.-Guaiene	36.765	0.193	--	--	--
1R,4R,7R,11R-1,3,4,7-Tetramethyltricyclo[5.3.1.0 (4,11) undec-2ene	37.476	0.156	--	--	--
Tricyclo[3.2.1.02,7]oct-3-ene, 2,3,4,5-tetramethyl-	38.041	0.899	--	--	--
trans-1,10-Dimethyl-trans-9-decalol (geosmin)	39.13	4.001	43.328	--	--
(4R,4aR)-4,4a-Dimethyl-6-(prop-1-en-2-yl)-1,2,3,4,4a,7-hexahydronaphthalene	39.373	0.286	--	--	--
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	39.598	--	--	--	1.514
2-Undecanone, 6,10-dimethyl-	39.844	--	--	--	0.825
(3S,3aS,6R,8aS)-3,7,7-Trimethyl-8-methyleneoctahydro-1H-3a,6-methanoazulene	42.23	--	--	--	0.946
(+)-epi-Bicyclosesquiphellandrene	42.725	0.153	--	--	--
(3R,5aR,9S,9aS)-2,2,5a,9-Tetramethyloctahydro-2H-3,9a-methanobenzo[b] oxepine	42.872	0.192	--	--	--
Ginsenosol	42.916	--	1.496	--	--
.gamma.-Muuroleone	43.741	--	0.19	--	--
Germacrene D	43.853	1.058	1.029	--	--
2H-3,9a.Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-	45.043	--	1.456	--	--
Naphtalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	45.897	--	0.507	--	--
Naphtalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	46.365	0.244	--	--	--
Naphtalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,8a.alpha.)]-	46.561	--	0.756	--	--
But-3-enal, 2methyl-4-(2,6,6-trimethyl-1-cyclohexenyl)-	46.738	--	0.291	--	--
Hexanoic acid, 3,5,5-trimethyl-, octyl ester	50.536	--	--	1.905	--
2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	52.995	--	0.311	--	--
1,8,11-Heptadecatriene, (Z, Z)-	54.627	--	--	1.129	4.386
Estra-1,3,5 (10)-trien-17-one, 3,4-bis[(trimethylsilyl) oxy]-	58.659	0.05	--	--	--

Table 2. Inhibition of radial growth (IRG) of common phytopathogenic fungi and oomycetes by pure volatile compounds

	Fungi			Oomycetes	
	<i>Alternaria alternata</i>	<i>Fusarium solani</i>	<i>Pytopythium vexans</i>	<i>Pythium aphanidermatum</i>	<i>Phytophthora capsici</i>
Dimethyl disulfide (positive control)	26.3±0.67 b	41.1±8.0 b	65.7±1.9 b	99.9±0.1 a	62.6±3.3 bc
<i>trans</i> -2-Hexenal	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
2-(Methylthio) ethanol	12.9±0.9 cd	1.1±0.5 c	4.6±1.3 g	0.9±1.7 e	45.1±5.6 e
2-Pentanone	13.9±1.7 c	1.3±0.9 c	14.8±2.5 f	14.2±4.7 d	53.6±2.7 cde
2,5- Dimethylfuran	6.4±2.7 d	6.4±1.1 c	26.4±4.3 de	56.9±3.5 b	58.1±5.5 bcd
α -Pinene	9.8±4.5 cd	5.4±0.7 c	10.8±3.6 fg	28.4±5.6 c	60.7±3.2 bcd
2-Methyl-3-pentanone	9.3±2.6 cd	1.7±1.1 c	19.1±5.3 ef	51.6±2.4 b	63.4±1.5 b
3-Methyl-2-butanol	7.7±2.4 cd	5.83±3.1 c	9.8±5.1 fg	3.5±1.9 e	51.6±2.3 de
Geosmin+2-Methylisoborneol	7.4±2.7 cd	6.2±4.4 c	34.4±0.8 cd	2.5±0.8 e	60.3±1.2 bcd
Valencene	9.5±3.0 cd	4.0±1.0 c	40.8±4.2 c	2.9±1.6 e	59.0±3.6 bcd

Mean values with same letters between columns are statistically similar±standard error

medium or head space, demonstrating that the effect of *Streptomyces* on pathogens was mediated by non-volatile and volatile compounds. These types of compounds can exert additive or synergistic effects on antimicrobial activity of microorganisms (Korpi et al., 2009; Sánchez-Ortiz et al., 2016). However, the antifungal VOCs of the *Streptomyces* genus have received little attention. In our study, the VOCs profile varied among *Streptomyces* species and even among strains of the same species. Unexpectedly, some of the characteristic VOCs of the *Streptomyces* genus (geosmin, 2-methyl-2-bornene, and 2-methylisoborneol) were not produced by all *Streptomyces* strains. These compounds were produced by the strains *S. cangkringensis* CIAD-CA07 and *S. misionensis* CIAD-CA27, but *S. misionensis* CIAD-CA27 produced 10 times more geosmin than *S. cangkringensis* CIAD-CA07. The strains CIAD-CA45 and CIAD-CA48 of *S. kanamyceticus* did not produce geosmin. These variations undoubtedly altered the antifungal activity of tested strains. Quecine et al. (2008) observed that the non-volatile compounds mediated antifungal activity of strains of the *Streptomyces* genus was highly dependent of the strain. Similarly, Guo et al. (2019) observed that the antifungal activity of some *Trichoderma* species (*T. harzianum*, *T. hamatum*, and *T. velutinum*) varied with the specie due differences in their VOCs profile, attributing these differences to genetic variability among tested species. Similar metabolic differences and activities have already been reported for several species and strains of *Streptomyces* and other genera (Scholler et al., 2002; Wei-Wei et al., 2008). In our study, several antifungal VOCs were identified in cultures of *Streptomyces* strains, including phenylethyl alcohol, (+)-epi-bicyclosquiphellandrene,

germacrene D, and 2-(methylthio) ethanol. Li et al. (2010) observed that the biosynthesis of phenylethyl alcohol in *S. globisporus* reduced the growing of *Penicillium italicum*. Wan et al. (2008) observed that phenylethyl alcohol and (+)-epi-bicyclosquiphellandrene from *S. platensis* inhibited the growth of the fungi *Sclerotinia sclerotium* and *Botrytis cinerea*. Fernando et al. (2005) hypothesized that 2-hexenal was part of the defense mechanism of plants against pathogens. 2-pentanone and germacrene D from *S. philanthi* RM-1-138 also exert antifungal effects (Boukaew et al., 2013; Rocha et al., 2012). Tested *Streptomyces* strains did not produce high quantities of sulfur-containing antifungal compounds (i.e. isobutyl isothiocyanate, dimethyl disulfide, and 2-(methylthio) ethanol, among others), which are highly produced by other antagonistic microorganisms (i.e. *Pseudomonas stutzeri* E25, *Stenotrophomonas maltophilia* CR71, and *T. virens*) (Angel et al., 2016; Rojas-Solís et al., 2018). Only the strain *S. kanamyceticus* CIAD - CA48 produced 2-(methylthio) ethanol, a sulfur-containing compound probably involved in the antifungal activity of this strain. Li et al. (2015) demonstrated that the chemical nature and abundance of VOCs emitted by microorganisms determine their antifungal activity.

The VOCs emitted by *Streptomyces* strains reduced the aerial growth of fungi and oomycetes in the range of 22.58 to 96.74%. This effect was probably caused by the diffusion of VOCs from *Streptomyces* strains into the culture medium and/or aerial mycelium, causing a reduction of aerial growth of pathogens (Wei-Wei et al., 2008). Boukaew et al. (2013) observed IRG values from 17.03 to 100% for dual confrontation assays between phytopathogenic fungi and

Streptomyces strains and attributed the inhibitory effect to VOCs of *Streptomyces*. The growth of aerial mycelium in cultures of some pathogens was more susceptible to VOCs of *Streptomyces* strains. This was the case of *A. alternata*, revealing the importance of VOCs in controlling plant diseases. Amini et al. (2016) also observed that the VOCs from *Streptomyces* species caused a higher reduction of *F. oxysporum* f. sp. *ciceris*. This susceptibility of fungi to volatile or non-volatile compounds depends on the antimicrobial mechanisms exerted by *Streptomyces* strains as well as to the capacity of pathogens to counteract the attack of antagonistic microorganisms (Duffy et al., 2003). In our study, the development of mycelium inside of the medium was observed in VOCs mediated confrontation assays. This behavior had not been reported previously in *in vitro* studies with antagonistic strains of *Streptomyces*. It could be caused by the presence of oxygen in agar pores or to the solubility of growth-promoting volatiles in the culture medium (Wei-Wei et al., 2008). Non-volatile compounds from *Streptomyces* strains reduced the growth of aerial mycelium of phytopathogens by more than 40%. Similarly, Ruanpanun et al. (2010) observed that the growth of pathogenic fungi was reduced from 21.7 to 87.4% by some actinomycetes. Evangelista-Martínez et al. (2020) also observed that the growth of phytopathogenic fungi was significantly reduced by some strains of the *Streptomyces* genus. These mechanisms of pathogen inhibition have been reported for other bacterial genera such as *Bacillus* and *Pseudomonas* (Chaurasia et al., 2005; Hernández-León et al., 2015; Raza et al., 2015). In our study, the strains CIAD-CA07 and CIAD-CA27 were highly effective for controlling the fungi *F. solani* and *A. alternata*, while the strains CIAD-CA45 and CIAD-CA48 controlled well the Oomycetes *P. capsici*, *P. vexans*, and *P. aphanidermatum* (Fig. 3). Considering that the cell wall of the oomycetes and fungi is mainly composed by cellulose and chitin, respectively, it can be hypothesized that the antifungal activity of the strains CIAD-CA07 and CIAD-CA27 was determined by the synthesis of chitinase while the antipathogenic activity of the strains CIAD-CA45 and CIAD-CA48 might be related to the biosynthesis of cellulase and glucanase (Duffy et al., 2003; Latijnhouwers et al., 2003).

The *trans*-2-hexenal completely inhibited mycelial growth of tested pathogens (IRG = 100%) (Table 2). Zhang et al. (2017) exposed cultures of *Penicillium cyclopium* to this compound and found a minimum inhibitory concentration of 160 $\mu\text{L}\cdot\text{L}^{-1}$ and a minimum fungicidal concentration of 320 $\mu\text{L}\cdot\text{L}^{-1}$. Our results suggest that both values could be lower for tested pathogens, because any of them recovered their normal growth rate after being sub-cultured in fresh medium in absence of the volatile. The antifungal action of this volatile is attributed to its capability to alter the structure of the cellular membrane and increase the leakage of potassium ions and other cellular components (Zhang

et al., 2017). Ma et al. (2019) associated the inhibitory effect of this volatile on *Aspergillus flavus* with the activation of early apoptosis, mitochondrial dysfunction, disruption of pyruvate metabolism, reduction of acetyl-CoA synthesis, and alterations on ATP synthesis and activity of dehydrogenases in the mitochondrion. Yang et al. (2019) found IRGs of 50% and 100% at concentrations of 10 $\mu\text{L}\cdot\text{L}^{-1}$ and 100 $\mu\text{L}\cdot\text{L}^{-1}$ of dimethyl disulfide, respectively. These values are higher than those found in our study for the same volatile. Rojas-Solís et al. (2018) attributed these variations to the differences in tolerance among strains of plant pathogens to VOCs as well as to differences in defense responses and mechanisms. The VOCs 2-pentanone, 2-(methylthio) ethanol, 2,5-dimethylfuran, α -pinene, 2-methyl-3-pentanone, 3-methyl-2-butanol, geosmin + 2-methylisoborneol, valencene also inhibited effectively the growth of pathogenic oomycetes (IRGs above of 50%), especially of *P. capsici*, as compared to the fungi. These VOCs are also emitted by other genera of antagonistic bacteria and fungi and are contained in some essential oils (Rouissi et al., 2013; Wang et al., 2013). Our values of IRGs demonstrated that the mixture of VOCs showed stronger antifungal and antioomycete properties than a single VOC. The evaluation of single VOCs, however allow to demonstrate if it possesses or not these properties.

CONCLUSION

Tested *Streptomyces* strains inhibited the growth of phytopathogenic fungi and oomycetes by biosynthesizing non-volatile and volatile compounds. The *Streptomyces* strains emitted several types of VOCs, including alcohols, aldehydes, ketones, esters, terpenes, terpenoids, and thioethers. The *trans*-2-hexenal completely inhibited the mycelial growth of tested pathogens. New VOCs with anti-phytopathogenic capabilities need to be identified, estimating their minimum inhibitory and fungicidal concentrations. Further studies are required to assess the antifungal activity of volatile mixtures and to confirm the efficacy of tested *Streptomyces* strains under open field conditions.

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Authors' contributions

Pérez-Corral and Rios-Velasco conceived the study. Pérez-Corral conducted the experiments. Data were analyzed by Pérez-Corral, Molina-Corral, Olivas-Orozco, Ornelas-Paz and Ruiz-Cisneros. The manuscript was written by Pérez-Corral and Rios-Velasco with support from Acosta-Muñiz,

Salas-Marina and Fernández-Pavía. The manuscript was reviewed by Ornelas-Paz and Ruiz-Cisneros. All authors read and approved the manuscript.

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